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(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US).

(72) Inventor: SADEE, Wolfgang; 125 Lagunitas, Ross, CA 94957 (US).

(74) Agents: ROBBINS, Billy, A. et al.; Robbins, Berliner & Carson, 201 North Figueroa Street, Los Angeles, CA 90012-2628 (US).

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(54) Title: COMPOSITIONS AND METHODS FOR ANTI-ADDICTIVE NARCOTIC ANALGESIS ACIVITY SCREENING AND TREATMENTS

(57) Abstract

The present invention provides assays to measure the regulation of the narcotic analgesic addictive state. Practice of the invention permits classification of test compounds for their effects on an activated opioid μ receptor state. When opioid μ receptor cells are treated with a test compositon under investigation, then in one embodiment the propensity of the test composition to elicit a spontaneous cAMP overshoot and an inverse agonist induced cAMP overshoot is determined and serves as a surrogate measure of addiction liability. The inverse agonist induced cAMP overshoot signifies the presence of what is designated as the constitutively active state for the opioid μ receptors. The use of these assays has led to the identification of compounds that have the desired effects on the constitutive activation of the opioid μ receptors. The therapeutic potential of these compounds include treating patients who are addicted to a narcotic analgesic or who have taken an overdose of a narcotic analgesic, or whose painis being relieved with a narcotic analgesic.

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COMPOSITIONS AND METHODS FOR ANTI-ADDICTIVE NARCOTIC ANALGESIC ACTIVITY SCREENING AND TREATMENTS

Field of the Invention

The present invention generally relates to screening for the pharmacological activities of narcotic drugs, and more particularly relates to assays useful in classifying the narcotic activity of compounds and treatments using anti-addictive agents determinable from such assays.

Background of the Invention

Endogenous opiate receptors were discovered in the 1970s, and have been intensely studied in seeking the mechanisms by which particular drugs lead to addiction. However, in his 1992 review of molecular mechanisms of drug addition, Nestler noted that such mechanisms have remained elusive. *J. Neurosci.*, *12* (7), pp. 2439-2450 (1992).

A number of different opioid receptor typ s have been identified. Among the known receptor types is the opioid μ receptor.

Narcotic analgesics act at the opioid μ receptor to produce analgesia. However, continued use of narcotic analgesics typically leads to habituation or addiction, and use of one leads to cross-tolerance/ dependence for the others. Despite their therapeutic uses, because patients develop increasing tolerances to the narcotic analgesics, increasing doses are required to achieve relief from pain. Undesirable side effects then tend to develop, such as physical dependence.

Illustrative narcotic analgesics are, for example, the various alkaloids of opium such as morphine, morphine salts (such as morphine hydrobromide, morphine hydrochloride, morphine mucate, morphine oleate, and morphine sulfate), and morphine analogs such as normorphine, diacetyldihydromorphine, codeine, and diacetylmorphine (heroin). Other widely used narcotic analgesics include methadone, meperidine, levorphanol, propoxyphene, fentanyl, oxymorphone, anileridine, metopon, and pentazocine.

The agonistic actions and dependence-producing properties of narcotic analgesics can be, and are, studied in various mammalian species besides humans, since practical and governmental considerations frequently require that studies be first done in small rodents and/or monkeys before the analgesic properties of pharmaceuticals are tested with humans. Drugs that have morphine-like properties in mammals other than man have been found to be morphine-like in man, and a variety of analgesic assays has been developed with animals which have gained widespread acceptance for predicting properties in humans. Biochemical changes during long term narcotic exposure can be studied in target tissues, such as the locus c eruleus (LC) of the rat, which is a good model of opiate dependency. Thus, upon chronic opiate treatment, researchers have demonstrated that LC neurons develop t lerance to the acute

inhibitory actions of opiates, and in parallel chronic treatment causes a dramatic upregulation of the cAMP second messenger system at every major step between receptor and physiological response, which leads to a dependent state. See, for example, Nestler at 2440.

To date, assays of the μ opioid receptor system have been unable to detect any major changes of that system during narcotic addiction. Thus, much of the current work has focused on events downstream of the receptor, such as long-term gene regulation, in attempting to account for the dependent state. Because the dependence liability of narcotic drugs severely limits their clinical utility as potent analgesics and exerts a heavy toll on society through illicit narcotic drug use, a screen for agents that could prevent or reverse the narcotic dependent state or might facilitate gradual withdrawal would greatly enhance the clinical utility of narcotic analgesics and could serve as an effective pharmacological weapon in the fight against illicit drug use.

Summary of the Invention

A novel mechanism is proposed where opioid μ receptor exposure to agonist leads to constitutive activation of those receptors. It is through this mechanism that the addictive state to narcotic analgesics is regulated.

One aspect of the present invention is to provide a means for assaying or measuring the regulation of the addictive state, in the search for compounds that prevent or reverse constitutive μ receptor activation, and to classify test compounds for their effects on th c nstitutively active μ receptor state.

Other aspects of the present inventi n are methods for treating patients who are addicted to a narcotic analgesic, or who have taken an overdose of a

narcotic analgesic, or whose pain is being relieved with a narcotic analgesic. Therapeutic methods in accordance with the invention normally involve selection of an agent with desired effects on the constitutive activation of opioid μ receptors. These desired effects are determinable from the inventive assays.

Accordingly, practice of the invention is expected greatly to enhance the clinical utility of narcotic analgesics and to serve as effective pharmacological weapons in the fight against illicit drug use.

Brief Description of the Drawings

Figure 1 is a schematic representation of relationships useful in understanding the tolerant dependent state.

Detailed Description of the Preferred Embodiments

An aspect of the present invention is to screen or classify test compounds with μ receptor activity for their effect on the constitutively active receptor. In order to practice the invention, effects on cAMP can be used as markers in one inventive embodiment.

Although the use of cAMP values is a preferred embodiment of the invention as indicia for determining opioid μ receptor activity, other effects and markers are also contemplated as being useful. For example, a ligand binding assay screen embodiment of the invention is faster than the cAMP assay screen since a ligand binding screen can rapidly test through a large number of compounds for their affinity to constitutively activated μ receptor sites.

With reference to Fig. 1 and when using cAMP values as indicia for opioid μ receptor activity, the constitutively active μ rec ptor is illustrated as "μ^*."

That is, the μ^* receptor represents the constitutively active state of the μ opioid receptor, whereas μ is the receptor in its resting state which is sensitive to stimulation by agonists. The cAMP system consists of a second messenger cascade with G proteins, adenylyl cyclase, and protein kinase A. Activated μ receptors generally inhibit the cAMP system, and the size of the arrows indicates the relative strength of this inhibition. In the naive state (no prior drug exposure), the activity of the μ^* state is minimal, and most receptors are drug sensitive. For purposes of illustration, morphine serves as a prototypal agonist, and naloxone (and CTOP also, as will be discussed hereinafter) as a classical antagonist, i.e., with no action by themselves but effective in blocking the agonist's effect on the resting μ state.

During development of the dependent state resulting from narcotic agonist pretreatment, a substantial upregulation of the cAMP system occurs, leading to a cAMP overshoot upon removal of the agonist (here referred to as "spontaneous cAMP overshoot"). In parallel, a slow net conversion of μ to μ^* occurs, so that there are fewer μ receptors remaining sensitive to the action of agonists, leading to tolerance. Further, the increased abundance of the μ^* state is essential to compensate for the upregulated cAMP system, to maintain close to normal cAMP levels. Hence, the hallmark of the tolerant-dependent state is the combination of the increased μ^* activity and the upregulated cAMP system. Naloxone is shown in Fig. 1 to act as an inverse agonist, i.e., it blocks the μ^* activity. Hence, the addition of naloxone to drug-free, tolerant-dependent tissu leads to an increased cAMP overshoot (here referred to as "naloxone cAMP overshoot"). In contrast, CTOP acts at the active μ^* recept r as a neutral or null antagonist by binding to μ^* without affecting activity.

In describing practice of this invention, a source of opioid μ receptors in combination with a means of monitoring constitutively active μ receptors, such as a cAMP system, will together sometimes hereinafter be termed the "biological system." A preferred source of opioid μ receptors that are exposed to or coupled with cAMP production is a human neuroblastoma (NB) cell line (SK-N-SH) and its NB subclone SH-SY5Y, both which express abundant μ opioid receptors (about 50,000 sites per cell). When intact cells are grown under appropriate cell culture conditions, the cells will be producing cAMP. Another source of a useful biological system for purposes of this invention can be certain tissues from experimental animals (e.g. rats and mice, which are good models for opioid μ receptor activity in humans), such rat locus coeruleus or guinea pig ileum.

When whole cells are used as the biological system, then it is desirable to add an adjuvant or stimulating agent of adenylyl cyclase, such as PGE, VIP, or forskolin, and to avoid phosphodiesterase inhibitors such as IBMX. Cells are preferably first differentiated with, for example, 1-10 μ M retinoic acid to enhance stimulatory and inhibitory receptor coupling to the cAMP system. Such preparations of a biological system have been described by Yu et al., *J. Neurochem.*, *51*, pp. 1892-1899 (1988); Yu et al., *J. Neurochem.*, *55*, pp. 1390-1396 (1990); and Yu and Sadée, *J. Pharmac. Exp. Ther.*, *245*, pp. 350-355 (1988).

When opioid μ receptor rich cells are treated with a test composition under investigation in accordance with the invention, then the propensity of the test composition to elicit the spontaneous and an inverse agonist induced cAMP overshoot can be determined and serve as a surrogate measure of addiction liability. The inverse agonist induced cAMP overshoot signifies the presence of constitutively active receptors.

In one variation of the assay, cells can be treated with a narcotic analgesic for 12 hours or longer to induce a dependent state, and then compounds or mixtures of compounds suspected as narcotic agonists or antagonists can be tested for their ability to mimic the inverse agonist induced cAMP overshoot or the agonist (e.g. morphine) caused depression of cAMP levels in the moderately tolerant cells. Control values are determined by measuring the effects of the receptors on cAMP production in the absence of agonist induced opioid μ receptor activity.

Test compounds that appear by themselves to have no effect on cAMP levels in drug free agonist pretreated-dependent cells should nevertheless then be tested in combination with either the agonist or the inverse agonist, in order to locate null antagonists. Compounds determined to be null antagonists (i.e. blocking the effects of morphine or of both morphine and naloxone with no effect when given alone) have the potential for treating overdoses of narcotic analgesics while avoiding the risk of excessive precipitated withdrawal, or they may serve to discourage additional drug uses by selectively blocking acute drug effects, with minimal long term effects (such as possible overall μ receptor upregulation by inverse agonists).

To summarize, one aspect of the present invention is an assay useful in screening for effects on opioid μ receptor activity. The assay can be performed by means of a kit that includes or is used in combination with a cAMP system. For example, when a cell line such as the SK-N-SH is used, then the cell line is capable of producing cAMP under cell growth conditions and is rich in opioid μ receptors. A first cAMP value is determined by measuring the effects of a first portion of these receptors on cAMP production in the absence of agonist induced opioid μ receptor activity.

This first cAMP value acts as a control value. Second and third cAMP values are also determined. The second cAMP value is determined by measuring the effects of a second portion of receptors on cAMP production while the receptors are in a constitutively active state but are substantially free of agonist molecules. The third cAMP value is determined by measuring the effects of the second portion of the receptors on cAMP production while they are in a constitutively active state, are substantially free of any agonist molecules, and are in the presence of a sufficient quantity of an inverse agonist to associate inverse agonist molecules with substantially all the receptors. In determining the third cAMP value, it is preferred to use a high concentration as feasible to associate inverse agonist molecules with substantially all the receptors, in order to achieve maximal effects (e.g. the highest cAMP overshoot).

The difference between the second cAMP value and the third cAMP value represents activity of the receptors. By "substantially free" of agonist molecules (in determining the second and third cAMP values) is meant there is less than about one percent of the total agonist drug remaining after pretreatment with a near maximally effective dose so that there would be no measurable effect in response curves. One can make the removal determinations through use of radioimmune assays or can look at the wash water by using the wash water to expose naive cells and determining by bioassays whether there is an effect. Typically by washing cells carefully three times, the substantial removal is accomplished if the agonist is morphine at 1 μM. If one is performing the assay *in vivo*, then the tissue is removed, sliced, and is washed in a water bath.

A variation of th assay permits the search for test compositions that prevent or decrease the formation of the constitutively activ μ receptor state

without affecting pharmacological potency. Such agents can be added to the opioid μ receptors during narcotic agonist incubation (to produce a constitutively active state) or after removal of the narcotic agonist, to test whether the overshoot induced by an inverse agonist can be reversed more rapidly. This class of test compounds has the potential to prevent or reverse the generation of constitutively active receptors and thus has the potential (when used therapeutically with a narcotic analgesic) to suppress the addictive liability of the narcotic analgesic, or may be useful by itself as an agent in treating narcotic drug addiction. Several such compounds (H7, H9, and HA-1004) have been identified using the proposed screen. In addition, H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) has been shown to reverse the tolerant-dependent state of morphine-injected mice to a native state, demonstrating the therapeutic potential of this class of compounds.

Recent evidence suggests that the constitutively active μ receptor state is formed by the phosphorylation of the μ receptor by a kinase belonging to a family of G protein-coupled receptor kinases (GRK). Thus, H7 and other such compounds are likely to be GRK inhibitors with other possible therapeutic uses. Moreover, an alternative method for screening for agents that prevent and/or reverse the formation of the constitutively active μ receptor state are standard enzyme activity assays for GRK such as one described by Chen and co-workers. Chen et al., *J. Biol. Chem.*, *268*, 7825-7831 (1993).

If cell membranes are the source of the desired biological system, then one typically will use the sam or similar pretreatments noted above, but will perform cAMP assays *in vitro* with the cell membranes.

Practice of the invention is generally useful in determining effects on opioid μ receptor activity, such as whether test compounds having opioid μ receptor activity would interact with the constitutively active μ receptor, or whether test compounds prevent or reverse the constitutively active μ receptor state. Practice of the invention permits classification of the ligand as a full inverse agonist, a partial inverse agonist, or a partial agonist. In addition, one can determine whether a test compound is a null (or neutral) antagonist.

By "full inverse agonist" is meant an agent that suppresses completely the effects of the constitutively active μ receptor state.

By "partial inverse agonist" is meant an agent that at maximal dosages suppresses only partially the effects of the constitutively active μ receptor state.

By "partial agonist" is meant an agent that at maximal dosages causes only partial activation of the resting, drug-sensitive μ receptor state.

By "null, or neutral, antagonist" is meant the compound simply binds to the receptor without changing its activity. A null antagonist may bind selectively to the resting, drug-sensitive μ receptor state, or to the constitutively active μ receptor state, or to both states.

Once agents are classified by means such as the inventive assay, optimal characteristics for treating drug addiction can be obtained in standard animal tests *in vivo*.

As will be further exemplified hereinafter, these classifications may be performed by determining certain cAMP values as reference points against which the cAMP effects of the test compound or composition are compared. That is, the first, second, and third cAMP values previously noted are used to classify the test compound or composition.

Receptors may show a certain minimal basal activity in the absence of agonist, and agonist exposure is usually thought to result in desensitization (e.g., by phosphorylation), and hence tolerance to a drug. However, it is proposed here that agonist exposure of a neurotransmitter receptors leads to constitutive activation, which no longer depends on the presence of an agonist. As the cAMP system becomes upregulated to compensate for the inhibitory influence of narcotics, the μ receptors become increasingly activated constitutively, i.e., no longer requiring an agonist. Thus, the μ^* receptors and the enhanced cAMP system balance each other out. When one assumes this type of activation occurs with the μ opioid receptor, many tolerance and dependence phenomena can be accounted for.

In cases where receptors are constitutively active, one distinguishes between agonists which further stimulate the remaining inactive receptors, and inverse agonists, which return the activated receptor to the inactive ground state. An example are the benzodiazepine receptors, where agonists are anxiolytic whereas inverse agonists are anxiogenic. Applied to the constitutively activated μ receptor in the dependent state, it is predicted that an inverse agonist will increase cAMP levels by reversing the active μ^* receptor state to the ground state. As will be further discussed below, naloxone is indeed such a reverse agonist, while it is also a classical antagonist of the resting, drug-sensitive μ receptor state.

Thus, for example, upon stimulation with morphine, the μ opioid receptors in SK-N-SH cells are gradually converted to a constitutively activated μ^* form which no longer depends on the presence of agonist. Maximal constitutive activation is expect d in th fully dependent state. After 12-48 hours pretreatment of SK-N-SH cells with 1 μM morphine, and th subsequent

complete removal of the drug, then naloxone significantly increases PGE_1-stimulated cAMP accumulation, whereas no increase or even a decrease is observed in cells treated with morphine for only 20 minutes or less. Therefore, naloxone acts as an inverse agonist at the constitutively activated μ opioid receptor (EC50~3 nM). These results are consistent with the observation that with increasing morphine dependence in experimental animals, the doses of naloxone required to elicit withdrawal symptoms are greatly reduced because of an increase of receptors in the active μ^* state.

With an upregulated cAMP system and a constitutively activated μ receptor, tolerance to narcotic agonists is expected because there are fewer receptors remaining to be activated. Further, one would expect lower doses of naloxone to precipitate withdrawal, because in the dependent state there are more activated receptors available for the inverse agonist to act on. Lastly, the decay of the activated μ receptor state would dictate the time course of withdrawal, rather than the rate of drug removal from the body. Residual constitutive receptor activity after the peak of withdrawal accounts for the continuing ability of naloxone to elicit overt withdrawal systems over a prolonged time period.

Such a constitutively activated μ opioid receptor mechanism goes beyond current hypotheses of narcotic addiction, and this mechanism lends itself to the discovery of agents that prevent or reverse constitutive activation, or that facilitate withdrawal by exhibiting the proper characteristics of a null antagonist or partial inverse agonist (to limit continued drug exposur without maintaining the d pendent state nor causing excessive withdrawal). Hence, compounds previously classified as mixed agonist-antagonist or partial agonist narcotic drugs may display

partial inverse agonism of potential utility in treating narcotic addiction. Knowledge of the mechanisms contributing to the regulation of the constitutively active receptor state could lead to diagnostic tests of individual drug dependence liability.

No compelling mechanism had been proposed previously that accounts for the driving force behind establishing and maintaining the narcotic dependent state. Even though an important role for the μ receptor in this process had long been speculated, experimental results have failed to reveal significant changes. Because the process of constitutive activation lends itself to screening anti-addictive agents and probing the molecular mechanisms of narcotic dependence, practice of the invention is expected to provide a new approach to separating the beneficial activity of narcotics from undesirable long term effects.

As earlier noted, the biological system being used for practicing the inventive assay can be pretreated, such as by treating cells with a narcotic analgesic for 12 hours or longer to induce a dependent state. The particular cell incubation and pretreatment conditions chosen will vary, with some relationships of treatment with cAMP determinations being summarized in Table 1.

TABLE 1

Pretreatment	- Washout -	Recovery	cAMP Assay	- cAMP Values
no drug	+	0-120 minutes	no drug	First cAMP value (control)
morphine	+	0-120 minutes	no drug	Second cAMP value (spontaneous cAMP over-shoot)
morphine	+	0-120 minutes	nalox-one	Third cAMP value (naloxone cAMP overshoot)

As suggested by Table 1, after the washout step and before the cAMP assay (with PGE_1), a recovery incubation stage of up to about two hours (where no drug is present) can be used during which one can test for reversal of the constitutively active μ receptor state.

With reference to Table 1 and using the "first cAMP value," "second cAMP value," and "third cAMP value" terminology previously described: the "first cAMP value" is the control level in untreated cells, the "second cAMP value" represents the spontaneous cAMP overshoot which rapidly drops to the control value if a recovery period of 30 minutes or more is used, and the "third cAMP value" is the naloxone cAMP overshoot above the spontaneous cAMP overshoot, which represents the constitutively active μ^* state. The "third cAMP value" remains elevated for at least two hours if a recovery incubation is used.

Practice of the invention has already proven its utility by permitting identification of a repre-

sentative compound that prevents constitutive activation of the μ receptor, and has also led to the identification of null antagonists.

Another aspect of the present invention is as a method of treating a patient suspected of having taken an overdose of a narcotic analgesic. Thus, one selects an agent determined to be a null antagonist for the suspected narcotic analgesic. The null antagonist will preferably have been determined as such by using the inventive assay.

As will be illustrated by Example 2, the determination of a null antagonist then permits administering a selected null antagonist for the suspected narcotic analgesic in a pharmaceutically effective amount, which means that the dose administered preferably is effective to block narcotic agonist effects in addicted patients without inducing severe withdrawal in treating a narcotic overdose or when one initiates withdrawal treatment. This is advantageous over present practice wherein naloxone is typically administered to patients suspected of having taken an overdose of a narcotic analgesic, which plunges the patient into an immediate severe withdrawal.

The pharmaceutically effective amount of agents determined to be null antagonists will be readily determinable clinically by establishing safe dosages and a dose-response curve in blocking analgesia in any established clinical pain model. Analgesia in rodent animal models can be measured by the tail-flick method of D'Amour and Smith, *J. Pharmac. Exp. Ther.*, *72*, pp. 74-79 (1941), and as modified by Tulunay and Takemori, *J. Pharmac. Exp. Ther.*, *190*, pp. 395-400 (1974), both inc rporated herein by reference. ED_{50} values, their 95% confidence limits, and significance of potency ratio betw en two ED_{50} values may be determined by the method

of Litchfield and Wilcoxon, *J. Pharmac. Exp. Ther.*, *96*, pp. 99-113 (1949), incorporated herein by reference.

Another aspect of the present invention is as a therapeutic method for treating a patient's pain. In this aspect of the present invention, an agent is determined to prevent constitutive activation of opioid μ receptors and/or to reverse constitutive activation of opioid μ receptors. This determination is preferably performed as will be described and exemplified by Example 1. The agent is then selected and administered in a therapeutically effective amount, such as in conjunction with a pain relieving amount of narcotic analgesic. That is, this aspect of the invention is directed to enhancing the clinical uses of narcotic analgesics because the agent selected prevents long term narcotic effects without blocking acute effects.

Alternatively, an agent shown to reverse constitutive μ^* receptor activation can be used to treat patients addicted to narcotic drugs. This agent would therefore remove the driving force of the dependent state and may thereby effectively treat narcotic addiction. Therapeutically effective amounts of the determined agents to be selected may be ascertained from dose-response curves in narcotic addicts where pretreatment with the agent would block subsequent naloxone induced withdrawal.

Aspects of the invention will now be further illustrated by specific examples, which are intended to exemplify the invention and not to be limiting thereof.

EXAMPLE 1

Because receptor activity is generally regulated by phosphorylation, several known protein kinase inhibitors were tested for their ability to prevent and reverse the formation of the constitutively

active μ receptor state in accordance with the invention, as follows.

The test compounds (10-100 μM) were first incubated with SK-N-SH cells alone (control) or together with 1 μM morphine during a 12 hour pretreatment period, followed by washout, no recovery period, and the cAMP assay (see Table 1), to establish the three cAMP values and thereby determine the spontaneous and naloxone induced cAMP overshoot. In a second set of experiments, the test compounds (10-100 μM) were added to the culture medium during a 30 minute or two hour recovery period. The first set of experiments was designed to identify agents that prevent the naloxone cAMP overshoot (i.e., prevent formation of the active μ^* state), whereas the second set of experiments was designed to identify agents that reverse the constitutive μ receptor activation in a short time period.

Among the compounds tested, H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (10 and 100 μM)) abolished the naloxone cAMP overshoot when added together with the morphine pretreatment for 12 hours, followed by complete removal of both morphine and H7 drugs. In contrast, H7 pretreatment for 12 hours did not prevent the acute depression of cAMP levels by morphine, showing that it does not interfere with the agonist induced activation of the resting μ receptor state. Furthermore, when added immediately after the 12 hour morphine pretreatment period, during a recovery period of 30-120 minutes, H7 completely reversed the naloxone cAMP overshoot, i.e. it reversed the constitutively active μ^* state to the resting μ state. Other compounds identified as protein kinase inhibitors capable of preventing formation of the constitutively active μ receptor state are H9 (N-(2-amino thyl)-5-isoquinolinesulfonamide dihydrochloride) and HA-1004 (N-(2-guanidino thyl)-5-isoquinolinesulfonamide hydro-

chloride). Because evidence suggests that this class of compounds are also GRK inhibitors, an alternative screening method are standard enzyme activity assays for GRK. See e.g. Chen et al., *J. Biol. Chem.*, *268*, 7825-7831 (1993).

H7 is known to inhibit several protein kinases including PKA and PKC. H7 is a representative of a class of compounds which could prevent and reverse long term narcotic effects by not contributing to the formation of the constitutively active μ receptor state but without blocking acute effects. This type of compound may be useful in enhancing the clinical use of narcotic analgesics or in treating narcotic addiction.

EXAMPLE 2

Another focus in practicing the inventive screening is to locate an opioid null antagonist with no ability to reverse the constitutively active μ opioid receptor state. Whereas naloxone is considered a μ opioid antagonist (i.e., blocking the activation of the μ receptor), it is also an inverse agonist, as defined here (i.e., blocking the constitutively active receptor) and illustrated earlier. Hence, its ability to cause severe and immediate withdrawal symptoms is high.

Using SK-N-SH cells, pretreated with 1 μM morphine for 12 hours, CTOP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) (1 μM) was found not to reverse the constitutive activity of the μ receptor state (i.e., it does not cause the naloxone cAMP overshoot), whereas it has previously been known to fully block the acute effects of morphine. In the inventive cAMP assay system, such a c mpound is now shown to block the acute eff cts of morphine, as expected from an antag nist at th r sting μ receptor state, but will also block the inverse agonist effects of naloxone.

To test for these properties of CTOP in the inventive assay, SK-N-SH cells were pretreated for 12 hours with 1 μM morphine (or with no drug as control) to establish the first and second cAMP values, with no recovery period before the cAMP assay (see Table 1). Then naloxone was replaced by CTOP (1-10 μM) to determine the third cAMP value. Since the second and third cAMP values were not different, CTOP does not act as an inverse agonist as does naloxone. To test whether CTOP blocks the effects of naloxone, CTOP (1-10 μM) and naloxone (0.1-1 μM) were added in combination to the cAMP assay. Reversal of the naloxone cAMP overshoot showed CTOP to act as a neutral (null) antagonist at the active μ^* state. Further, CTOP (1-10 μM) also reversed the reduction of the cAMP level caused by morphine (1 μM), confirming it to act as an antagonist at the resting μ state. Similar results were obtained with CTOP analog CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) and with nalorphine.

The selective μ antagonist CTOP, with a structure completely different from naloxone, is thus a prototypic example of a null antagonist of the μ receptor, having no effect on the constitutively active μ receptor state. The potential use of such a null antagonist is twofold. First, it could serve as an antagonist given clinically to counteract narcotic overdose, with the advantage over naloxone that immediate severe withdrawal is avoided (assuming that withdrawal results to a large degree from reversal of the constitutive μ receptor activity). Second, null antagonists may also be useful in treating or inducing treatment of narcotic addicti n, for exampl , in combination with a compound such as H7, to block the vicious circle of the dependent state.

Referring again to Fig. 1, the peptide μ opioid antagonist CTOP is shown here to act as a null or neutral antagonist at the μ^* receptor. Therefore, CTOP not only blocks the effects of the agonist morphine at the μ state, but also the effects of the inverse agonist naloxone at the activated μ^* state. The therapeutic potential of neutral antagonists is illustrated by the experiment showing CTAP causing significantly less withdrawal in morphine dependent mice and further reduced naloxane induced withdrawal.

Further applications for practice of the invention are to locate narcotic drugs with self-limiting maximal activity. Several narcotic agents display bell-shaped dose-response curves. These drugs produce maximal effects at an intermediate dosages level, and at higher doses reverse their own effects. The mechanism of this behavior of opioid drugs is unknown. Partial agonists at the μ receptor could at high doses also act as inverse agonists at the constitutively activated μ receptor, thereby blocking their own effects. The potency of the agonist and inverse agonist properties must be balanced such that sufficient acute effects are attained, and maximal effects, associated with respiration depression, are blunted.

Target compounds as safe analgesics are μ (partial) agonists with sufficient potency and efficacy at the constitutively active μ receptor to limit maximal response and side effects such as respiratory depression. Buprenorphine, a clinically used analgesic, is a prototypic example of such a compound although its self-limiting properties are insufficient to prevent respiratory depression and addiction liability. Null antagonists acting at th constitutively active μ receptor only identified in this fashion could also serv in combination with conventional agonists to minimize peak effects or limit th duration of action.

As earlier noted, one need not use the cAMP system as the sole indicator of effects on the opioid μ receptor in practicing this invention. With the use of ^3H labeled opioid tracers, one can alternatively rapidly screen for agents with an ability to bind to the constitutively active μ receptor by the ligand binding assay aspect of this invention. Narcotic agonists (e.g. morphine, DAMGE) have very low affinity to the constitutively active μ receptor and can serve as an analytical tool to block inactive μ receptors, thereby allowing selective labeling of constitutively active μ sites, e.g., with ^3H-naloxone or ^3H-CTOP, in the tolerant-dependent tissue where the presence of the μ^* state is shown to be dramatically increased. Thus, a ligand binding assay in accordance with the invention comprises providing a plurality of opioid μ receptors of which at least some (preferably most) are in a constitutively active state. Any inactive receptors are blocked with a narcotic agonist. The receptors are placed in a constitutively active state when treated with a narcotic analgesic for a sufficient time and at a sufficient concentration. Any inactive receptors are blocked with narcotic antagonist. Thus, one is able to selectively label the constitutively active μ sites, such as with radioactive atoms, preferably tritium labeled opioid tracers. Then when the receptors are exposed to a test composition, one is able to determine whether the test composition binds to the selectively labeled receptors. The selective labelling with, for example, radioactive tracers is preferably accomplished by an incubation, usually conducted within a temperature range of about 20°C-37°C. Thus, when one adds the test composition, a conventional competitive binding assay can be performed to determine binding affinity. Such a ligand binding assay screen embodiment is faster than the cAMP assay screen embodiment when testing through a large number of

compounds for their affinity to constitutively active μ sites.

EXAMPLE 3

The proposed model of narcotic tolerance and dependence should be a general phenomenon, applicable to all tissues containing the μ opioid receptor. The guinea-pig ileum is one of the most widely used *in vitro* tissue preparations, where narcotic agonists inhibit electrically induced twitching, as the functional endpoint. Very brief exposure to morphine (~5 min) is sufficient to produce a dependent state which is characterized by naloxone induced twitching (after morphine has been completely removed). This naloxone induced twitching response is the equivalent to the *naloxone cAMP overshoot* in morphine pretreated SH-SY5Y cells.

The guinea-pig ileum was used to test the effects of kinase inhibitors and neutral antagonists. As predicted from the proposed mechanism of μ^* formation, treatment with H7 (50 μM) and several analogs (H9, HA-1004, H8) largely suppressed naloxone induced twitching in the dependent guinea-pig ileum, suggesting prevention of μ^* formation. Moreover, the proposed neutral μ receptor antagonist CTOP produced no twitching in the dependent guinea-pig ileum, but is suppressed naloxone induced twitching. These results were all predicted from the behavior of these compounds in SH-SY5Y cells, providing strong evidence for the notion that constitutive activation of the μ receptor is crucial to the dependent state. Further, organ tissues such as the guinea-pig ileum, or the mouse vas deferens, are also suitable for screening anti-addictive agents, such as kinase inhibitors and neutral antagonists.

EXAMPLE 4

Recently, several laboratories have cloned the μ receptor gene (e.g. Chan et al., *Molec. Pharmacolo.*, *44*, 8-12 (1993)). We have stably transfected the μ gene into U293 cells (U293-μ ~10^6 sites/cell), for a more convenient and definitive detection of μ^* activity for use as a screen for anti-addictive agents. Morphine pretreatment and washout of the drug were the same as described for Sh-SY5Y cells. Stimulation of cAMP accumulation in this case was best done with 10 μM forskolin for 10 minutes. Under these conditions, there was a measurable *spontaneous cAMP overshoot* after morphine pretreatment; however, the addition of 1-10 μM naloxone caused a very large additional increase in cAMP levels (500-600% ≡ *naloxone cAMP overshoot*), which indicates the presence of substantial μ^* activity suppressing cAMP levels. These results show that the narcotic tolerant-dependent state can be reproduced in transfected non-neuronal cells, which can therefore serve as an attractive screening method for identifying anti-additive drugs.

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

It is Claimed:

1. A kit useful in screening for effects on opioid μ receptor activity when in combination with a cAMP system comprising:

a plurality of opioid μ receptors;

a first cAMP value, the first cAMP value determinable by measuring the effects of a first portion of the receptors on cAMP production in the absence of agonist induced opioid μ receptor activity;

a second cAMP value, the second cAMP value determinable by measuring the effects of a second portion of the receptors on cAMP production while in a constitutively active state but being substantially free of agonist molecules; and,

a third cAMP value, the third cAMP value determinable by measuring the effects of the second portion of the receptors on cAMP production while (a) in a constitutively active state, (b) being substantially free of any agonist molecules, and (c) in the presence of a sufficient quantity of an inverse agonist to associate inverse agonist molecules with substantially all the receptors.

2. A cell line kit useful in screening for effects on opioid μ receptor activity comprising:

a cell line capable of producing cAMP under cell growth conditions and having opioid μ receptors;

a first cAMP value, the first cAMP value determinable by measuring the effects of a first portion of the receptors on cAMP production in the absence of agonist induced opioid μ receptor activity;

a second cAMP value, the second cAMP value determinable by measuring the effects of a second portion of the receptors on cAMP production while in a

constitutively active state but being substantially free of agonist molecules; and,

a third cAMP value, the third cAMP value determinable by measuring the effects of the second portion of the receptors on cAMP production while (a) in a constitutively active state, (b) being substantially free of any agonist molecules, and (c) in the presence of a sufficient quantity of an inverse agonist to associate inverse agonist molecules with substantially all the receptors.

3. The kit as in claim 2 wherein the cell line is SK-N-SH cells or SH-SY5Y cells.

4. The kit as in claim 2 wherein the cell line is U293 cells where the μ gene has been stably transfected.

5. An assay useful in screening for effects on opioid μ receptor activity comprising:

providing a plurality of opioid μ receptors exposed to a cAMP production system;

determining a first cAMP value by measuring the effects of a first portion of the receptors on cAMP production in the absence of agonist induced opioid μ receptor activity;

determining a second cAMP value by measuring the effects of a second portion of the receptors on cAMP production while in a constitutively active state, but being substantially free of agonist molecules;

determining a third cAMP value by measuring the effects of the second portion of the receptors on cAMP production while (a) in a constitutively active state, (b) being substantially free of any agonist molecules, and (c) in the presence f a sufficient quantity of an inverse agonist or a test compound to

associate inverse agonist or test compound molecules with substantially all the receptors; and

combining a test portion of the receptors with a test composition and measuring the effects thereof on cAMP production as a test cAMP value.

6. The assay as in claim 5 wherein the plurality of opioid μ receptors exposed to a cAMP production system are provided as viable cells and include an adenylyl cyclase stimulating agent.

7. The assay as in claim 6 wherein the cells are SK-N-SH or SH-SY5Y.

8. The assay as in claim 6 wherein the cells are U293 where the μ gene has been stably transfected.

9. The assay as in claim 6 wherein the cells are U293 where the μ gene has been stably transfected and the adenylyl cyclase stimulating agent is forskolin.

10. The assay as in claim 5 further comprising:

comparing the test cAMP value against one or more of the first cAMP value, the second cAMP value, and the third cAMP value.

11. The assay as in claim 5 further comprising:

classifying the test composition as a partial agonist, a null agonist, a partial inverse agonist, or a full inverse agonist by comparing the test cAMP value against on or more of the first cAMP value, the second cAMP value, and the third cAMP value.

12. The assay as in claim 5 wherein the difference between the second cAMP value and the third cAMP value represents activation of the receptors when in a constitutively active state.

13. A ligand binding assay useful for determining effects on opioid μ receptors comprising:

providing a plurality of opioid μ receptors, at least some of the receptors being in a constitutively active state with any inactive receptors being blocked with a narcotic agonist; and,

selectively labelling the constitutively active receptors.

14. The assay as in claim 13 wherein the labelling is performed with radioactive atoms.

15. The assay as in claim 13 further comprising:

exposing the receptors to a test composition.

16. The assay as in claim 15 further comprising:

determining whether the test composition binds to the selectively labelled receptors.

17. The assay as in claim 13 wherein the blocking narcotic agonist is morphine or DAMGE.

18. The assay as in claim 13 wherein the opioid μ receptors are constitutively activated by incubation with a narcotic analgesic.

19. Th assay as in claim 14 wherein the lab lling is performed with a compound including tritium.

20. The assay as in claim 19 wherein the labelling uses ^{3}H-naloxone or ^{3}H-CTOP.

21. The assay as in claim 19 wherein the labelling uses ^{3}H-CTAP or ^{3}H-D-Tic-CTAP or ^{3}H-nalorphine

22. A method of treating a patient suspected of having taken an overdose of a narcotic analgesic, comprising:

selecting an agent determined to be a null antagonist for the suspected narcotic analgesic; and,

administering the selected null antagonist for the suspected narcotic analgesic in a pharmaceutically effective amount.

23. The method as in claim 22 wherein the dose administered is effective to block narcotic agonist effects in addicted patients without inducing severe withdrawal in treating a narcotic overdose or to initiate withdrawal treatment.

24. The method as in claim 22 wherein the null antagonist administered includes D-Phe-Cys-Tyr-D-Trp-Orn-Tyr-Pen-Thr-NH_2.

25. The method as in claim 22 wherein the null antagonist administered is nalorphine.

26. The method as in claim 22 wherein the null antagonist administered is CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2).

27. The method as in claim 22 wherein the null antagonist administer d is D-Tic-CTAP (D-Tic-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2).

28. A therapeutic method for treating a patient's pain, comprising:

selecting an agent determined to prevent constitutive activation of opioid μ receptors; and,

administering a therapeutically effective amount of the selected agent in conjunction with a pain relieving amount of narcotic analgesic.

29. The therapeutic method as in claim 28 wherein the agent selected is 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride, and the narcotic analgesic is morphine.

30. The therapeutic method as in claim 28 wherein the agent selected is H9 (N-(2-aminoethyl)-5-isoquinolinesulfonamide dihydrochloride).

31. The therapeutic method as in claim 28 wherein the agent selected is HA-1004 (N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride).

32. A therapeutic method for treating a patient addicted to a narcotic drug, comprising:

selecting an agent determined to prevent and/or reverse constitutive activation of opioid μ receptors; and

administrating a therapeutically effective amount of the selected agent to the addicted patient.

33. The therapeutic method as in claim 32 wherein the agent selected is 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride.

34. The therapeutic method as in claim 32 wherein the agent selected is H9 (N-(2-aminoethyl)-5-isoquinolinesulfonamide dihydrochloride).

35. The therapeutic method as in claim 32 wherein the agent selected is HA-1004 (N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride).

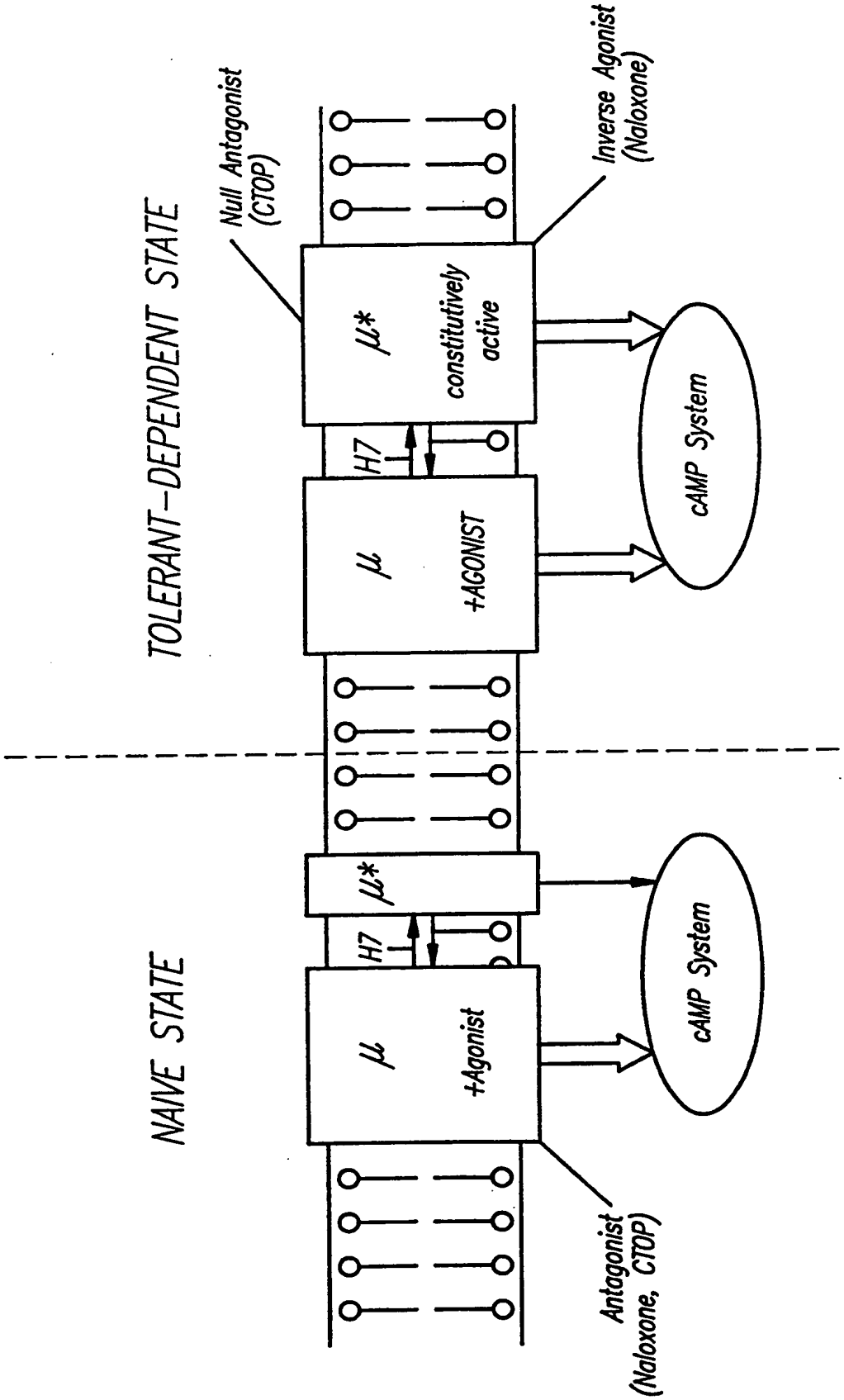

FIG. 1

INTERNATIONAL SEARCH REPORT

L. national application N .
PCT/US94/06883

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/554; A61K 31/47, 37/02
US CL :435/7.21; 514/2, 311
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.21; 436/501; 514/2, 311

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, DIALOG, APS
search terms: opioid receptors, morphine, nalaxone, cAMP, CTOP

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	JOURNAL OF NEUROCHEMISTRY, VOLUME 55, ISSUED 1990, YU ET AL., "REGULATION OF CYCLIC AMP BY THE μ-OPIOID RECEPTOR IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS", PAGES 1390-1396, SEE ENTIRE ARTICLE.	1-3 ---- 5-12
Y	EUROPEAN JOURNAL OF PHARMACOLOGY, VOLUME 198, ISSUED 1991, ABDELHAMID ET AL., "CHARACTERISTICS OF μ AND δ OPIOID BINDING SITES IN STRIATAL SLICES OF MORPHINE-TOLERANT AND -DEPENDENT MICE", PAGES 157-163, SEE ABSTRACT.	13-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date f mailing of the international search report
06 AUGUST 1994	16 AUG 1994
Name and mailing address f the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized fficer LORA M. GREEN Teleph ne No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06883

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, VOLUME 248, NUMBER 1, ISSUED 1989, HAWKINS ET AL., "[^{3}H]-[H-D-PHE-CYS-TYR-D-TRP-ORN-THR-PEN-THR-NH_2] ([^{3}H]CTOP), A POTENT AND HIGHLY SELECTIVE PEPTIDE FOR *MU* OPIOID RECEPTORS IN RAT BRAIN", PAGES 73-80, SEE ABSTRACT.	22-24 -------- 13-21, 25-27
X -- A	US, A, 5,061,715 (SUNKARA ET AL.) 29 OCTOBER 1991, COLUMN 2, LINES 1-60.	32-35 -------- 28-31
X -- Y	MOLECULAR PHARMACOLOGY, VOLUME 44, ISSUED 1993, CHEN ET AL., "MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF A μ-OPIOID RECEPTOR FROM RAT BRAIN", PAGES 8-12, SEE ABSTRACT.	4 --- 8, 9
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, VOLUME 72, NUMBER 8, ISSUED AUGUST 1975, SHARMA ET AL., "DUAL REGULATION OF ADENYLATE CYCLASE ACCOUNTS FOR NARCOTIC DEPENDENCE AND TOLERANCE", PAGES 3092-3096, SEE PAGE 3094, SECOND COLUMN.	5-12